



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ :

A61K 9/50, 9/52, 47/24

A61K 47/42, 47/46, 47/48

A1

(11) International Publication Number:

WO 92/17167

(43) International Publication Date:

15 October 1992 (15.10.92)

(21) International Application Number: PCT/AU92/00141

(22) International Filing Date: 2 April 1992 (02.04.92)

(30) Priority data:

PK 5385

2 April 1991 (02.04.91)

AU

(71) Applicant (for all designated States except US): BIOTECH AUSTRALIA PTY. LTD. [AU/AU]; 28 Barcoo Street, East Roseville, NSW 2069 (AU).

(72) Inventors; and

(75) Inventors/Applicants (for US only) : RUSSELL-JONES, Gregory, John [AU/AU]; 23 Greenfield Avenue, Middle Cove, NSW 2068 (AU). WESTWOOD, Steven, William [AU/AU]; 6/98 Alt Street, Ashfield, NSW 2131 (AU).

(74) Agent: SPRUSON & FERGUSON; GPO Box 3898, Sydney, NSW 2001 (AU).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published

With international search report.

(54) Title: ORAL DELIVERY SYSTEMS FOR MICROPARTICLES

(57) Abstract

There are disclosed complexes and compositions for oral delivery of a substance or substances to the circulation or lymphatic drainage system of a host. The complexes of the invention comprise a microparticle coupled to at least one carrier, the carrier being capable of enabling the complex to be transported to the circulation or lymphatic drainage system via the mucosal epithelium of the host, and the microparticle entrapping or encapsulating, or being capable of entrapping or encapsulating, the substance(s). Examples of suitable carriers are mucosal binding proteins, bacterial adhesins, viral adhesins, toxin binding subunits, lectins, Vitamin B₁₂ and analogues or derivatives of Vitamin B₁₂ possessing binding activity to Castle's intrinsic factor.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark			US	United States of America

ORAL DELIVERY SYSTEMS FOR MICROPARTICLES**TECHNICAL FIELD**

The present invention relates to complexes and compositions for oral
5 delivery of a substance(s) to the circulation or lymphatic drainage system of a
host. The invention also relates to processes for the production of complexes
and compositions for oral delivery of a substance(s) to the circulation or
lymphatic drainage system of a host. The invention further relates to a method
of delivering a substance(s) to the circulation or lymphatic drainage system of
10 a host. In addition the invention relates to kits for preparing complexes for oral
delivery of a substance(s) to the circulation or lymphatic drainage system of a
host.

BACKGROUND ART

A number of clinical conditions of vertebrates have sufficiently
15 deleterious effects upon the vertebrate to warrant the administration of some
pharmaceutically active agent. Such agents may include (i) vaccines, to
protect against diseases such as tetanus, diphtheria or whooping cough, (ii)
hormones, e.g. Insulin, LHRH, vasopressin, oxytocin, or (iii) drugs, e.g. anti-
cancer agents, antibiotics. In these cases, a suitable agent is administered to
20 the vertebrate to invoke immunity, to supplement hormone levels, to eliminate
the disease causing agent or to provide a therapeutic effect.

Administration of the pharmaceutical to the vertebrate may be via a
number of routes including intramuscular (i.m.), subcutaneous (s.c.), or oral
(per os, p.o.) administration. I.m. or s.c. administration of the pharmaceutical
25 suffers from the disadvantages that: relatively specialized skills are required to
administer the pharmaceutical; large scale administration may be difficult to
perform; it is expensive; and a number of side reactions can occur to the
agent being administered. For these reasons oral administration of the
pharmaceutical is generally preferred. Many antibiotics (tetracycline, penicillin
30 etc), and hormones (progesterone, oestrogen etc) can be successfully
administered via the oral route. There are however drugs, hormones and
immunogens whose efficacy is almost totally lost upon oral administration
(including Calcitonin, Erythropoietin, Granulocyte Colony Stimulating Factor,
Stem Cell Factor, Granulocyte Colony Stimulating Factor, LHRH analogues,
35 Somatostatin, Insulin, Interferons, Plasminogen Activator Inhibitors and
species of DNA and RNA). This loss of efficacy may be due either to the
inability of the intestinal mucosa to absorb these compounds or the breakdown
of these substances by various physiological agents in the intestinal milieu. To
some extent this effect can be overcome by the administration of extremely

large doses of the pharmaceutical agent. This approach, however, is not economically feasible for many pharmaceutical agents.

In an attempt to overcome the problem of degradation a number of encapsulation methods have been employed which enable the encapsulated material to by-pass both the gastric acidity and the pepsin mediated proteolysis encountered within the lumen of the stomach. Typically these methods have involved enteric coated capsules, which only release their contents upon contact with the higher pH of the upper duodenum and jejunum. While this has greatly increased the oral efficacy of a number of compounds, still many substances are pharmaceutically inactive upon oral delivery and must be administered parenterally. Noteable examples of such compounds include Calcitonin, Erythropoietin, Granulocyte Colony Stimulating Factor, Stem Cell factor, Granulocyte Macrophage Colony Stimulating Factor, Somatostatin, Insulin, LHRH and its analogues, Interferons, Plasminogen Activator Factor, species of DNA and RNA, and many vaccines.

In a further extension of the encapsulation process, several new forms of encapsulation have been designed in recent years with the specific purpose of trapping large quantities of pharmaceuticals in subcellular capsules, in the hope that once protected from the intestinal milieu, the capsules would themselves be taken up from the intestine and release their contents systemically. Two basic forms of these capsules have been developed, nanocapsules (or microcapsules) and nanospheres (or microspheres). In essence these particles can be formed by one of a number of methods, several of which are outlined below:

(i) Solvent Evaporation

In which a compound which is soluble in one solvent is dispersed into a non-miscible solvent and the first solvent is evaporated off. Particles formed in this fashion have been used to administer (parenterally) a number of water insoluble compounds. An example of such a system would be the formation of polyalkylcyanoacrylate nanocapsules in which the antifungal agent, griseofulvin is entrapped.

(ii) Desolvation

In this method a compound is contained in a liquid in which it is soluble (the solvent) and a second liquid (which is miscible with the first liquid, but in which the compound is not soluble) is added to the solvent. As more of the second liquid is added the compound becomes desolvated. During the process of desolvation the compound rich phase (the coacervate) contains an enriched amount of compound which is dispersed as microdroplets in the compound deficient phase. At this stage the coalesced material can be chemically

crosslinked by a suitable crosslinking agent to form micro or nano-particles. Nanoparticles of gelatin or BSA can be prepared in this way. Solutions of these proteins are desolvated by the addition of sodium sulfate, or ammonium sulfate solutions. At the point of desolvation there is an increase in turbidity, at which time the nanoparticles can be formed by the addition of a suitable cross-linker such as glutaraldehyde or butanedione.

(iii) Complex coacervation

In this procedure two polyelectrolytes having opposite charge are mixed in aqueous medium so that a spontaneous liquid/liquid phase separation occurs. The phenomenon is limited to polymers having a suitable ionic charge density and chain length. Typically these microspheres are formed by the addition of a polyanion such as Gum Arabic, Alginate, or Polyphosphate, to a polycation such as Gelatin.

(iv) Polymer/polymer incompatibility

This procedure is based upon the observation that two chemically different polymers dissolved in a common solvent are usually incompatible. Thus the mixture will tend to form two phases. The insoluble phase can be used to coat core particles to form microcapsules. An example would be the precipitation of ethyl cellulose from cyclohexane by the addition of polyethylene.

(v) Interfacial Polymerization

In this technique, two reactants, each dissolved in a mutually immiscible liquid, diffuse to the interface between the two liquids where they react to form a capsule wall. An example of such capsule formation would occur if a mixture of Sebacyl chloride dissolved in an oil phase was emulsified into an aqueous phase containing ethylenediamine.

Oppenheim and coworkers (1982) have used the desolvation process (described above) to prepare insulin nanoparticles. These nanoparticles were found to be highly effective when administered intravenously, however a disappointingly small quantity of insulin was delivered to the systemic circulation when these particles were given orally. It would appear, from this work that although it was possible to protect the insulin from degradation in the intestine it was not possible to target the nanoparticles to the intestinal mucosa in such a way as to cause uptake. The lack of a suitable targeting agent has in fact rendered this type of microencapsulation technique to be generally unsuitable for oral delivery of encapsulated agents.

Recent work in part undertaken by one of the current inventors (W086/06635 and PCT/AU86/00299, the disclosures of which are

incorporated herein by reference) has, however, provided such a targetting mechanism. In this work use was made of two natural uptake mechanisms in the gut. The first mechanism utilizes the natural uptake mechanism for Vitamin B₁₂. During this uptake Vitamin B₁₂ firstly binds to intrinsic factor (IF) in the upper small intestine. The Vitamin B₁₂-IF complex then passes down the small intestine and binds to an IF receptor located on the surface of the ileal epithelium. The whole Vitamin B₁₂-IF-receptor complex is then internalized by receptor-mediated endocytosis and some time later the Vitamin B₁₂ appears in the serum. It has been shown that it is possible to chemically link peptides to Vitamin B₁₂ in such a manner that does not interfere with its complexing to IF, and to deliver these molecules to the circulation following oral administration. The use of Vitamin B₁₂ as a carrier for the oral delivery of active substances is described in PCT/AU86/00299.

In the second mechanism, natural mucosal binding proteins were employed to target various haptens and protein molecules to the gastrointestinal mucosa and elicit their uptake. These binding proteins included bacterial adhesins (987P and K99 pili), a viral adhesin (flu virus), a toxin binding subunit (LTB), as well as a number of plant lectins. This class of molecules was termed carrier molecules.

Both the above described mechanisms do however suffer from the disadvantage that the amount of material which could be delivered through either uptake mechanism was directly proportional to the amount of targetting agent which could be taken up. In this regard, the vitamin B₁₂ uptake mechanism is limited by the absolute quantity of Vitamin B₁₂ which is normally absorbed, which in most animals amounts to only a few micrograms.

Furthermore, in order for either carrier system to work effectively the conjugated material (hormone, peptide or drug) must preferably be able to survive the proteolytic environment of the small intestine and must also contain a suitable site for chemical cross-linkage to the carrier. During the conjugation, care must be taken to preserve the pharmacological activity of the active agent both during the conjugation as well as in the final complex. Furthermore, a number of peptides may not be suitable for oral delivery (due to sensitivity to proteolysis, or due to lack of suitable functional groups for conjugation) and so new analogues may need to be developed which possess an appropriate conjugation site or have been designed to resist proteolytic degradation. In this respect the present invention can be distinguished from the previous inventions described above in that the carrier molecule of the present invention is not covalently conjugated to the pharmaceutically active agent, but rather the carrier molecule is either covalently linked to the

material/polymer comprising the microsphere, or is associated hydrophobically with the surface of the microsphere during its formation.

Surprisingly, the present inventors have discovered that it is possible to prepare complexes comprising at least one carrier molecule and at least one microparticle comprising an active pharmaceutical agent. More surprisingly, the present inventors have discovered that the carrier in such complexes can enable the complex comprising a relatively large microparticle to be transported to the circulatory or lymphatic drainage system via the mucosal epithelium of a host. Thus, the present invention overcomes the above-described disadvantages of the methods of oral delivery of the prior art, since in the complexes of the present invention the active agent is not chemically modified and its physiological activity is preserved while the microparticle provides a protection against degradation or modification in the gastrointestinal environment. Furthermore, the microparticles of the invention are linked to a carrier molecule which can specifically target the microparticles to the intestinal epithelium and provoke uptake.

Other advantages of the present invention will be apparent from the objects of the invention and the disclosure of the invention hereinbelow.

OBJECTS OF INVENTION

Objects of this invention are to provide complexes and compositions for oral delivery of a substance(s) to the circulation or lymphatic drainage system of a host.

Other objects are to provide processes for the production of complexes and compositions for oral delivery of a substance(s) to the circulation or lymphatic drainage system of a host, a method of delivering a substance(s) to the circulation or lymphatic drainage system of a host and kits for preparing complexes for oral delivery of a substance(s) to the circulation or lymphatic drainage system of a host.

DISCLOSURE OF INVENTION

The term "carrier" as used throughout the specification includes mucosal binding proteins, Vitamin B₁₂, and analogues or derivatives of Vitamin B₁₂ possessing binding activity to Castis's intrinsic factor, and also includes within its meaning the expression "carrier molecule".

The term microparticle as used throughout the specification includes microspheres and microcapsules and refers a small particle ranging in size from 1 nanometer to 100 micrometers in diameter.

According to a first embodiment of this invention there is provided a complex for oral delivery of a substance to the circulation or lymphatic drainage system of a host, comprising:

a microparticle coupled to at least one carrier;

the carrier being capable of enabling the complex to be transported to the circulation or lymphatic drainage system via the mucosal epithelium of the host;

5 the microparticle entrapping or encapsulating the substance whereby the substance is substantially unaffected by intestinal digestive substances of the host; and

the microparticle being adapted to release the entrapped or encapsulated substance into the circulation or lymphatic drainage system of
10 the host.

A particularly desired form of the complex of the first embodiment of the present invention is a microsphere or microcapsule coupled to a carrier molecule, the microsphere or microcapsule enclosing a hormone, drug, immunogen, or DNA or RNA (such as ribozyme) component, molecule or
15 analogues thereof, wherein the carrier molecule is a mucosal binding protein or Vitamin B₁₂, or an analogue or derivative of Vitamin B₁₂ possessing binding activity to Castle's intrinsic factor.

According to a second embodiment of this invention there is provided a complex for oral delivery of a substance to the circulation or lymphatic
20 drainage system of a host, comprising:

a microparticle coupled to at least one carrier;

the carrier being capable of enabling the complex to be transported to the circulation or lymphatic drainage system via the mucosal epithelium of the host;

25 the microparticle being capable of entrapping or encapsulating the substance whereby the substance is substantially unaffected by intestinal digestive substances of the host; and

the microparticle being adapted to release the entrapped or encapsulated substance into the circulation or lymphatic drainage system of
30 the host.

In the first and second embodiments each microparticle may have a single carrier coupled to it.

Alternatively, in the first and second embodiments a plurality of carriers which may be the same or different may be coupled to the microparticle.
35 Alternatively, a plurality of microparticles which may be the same or different and which may contain the same substance or different substances may be coupled to the carrier. Typically, the plurality of carriers is from 2 to 100000, generally from 2 to 10 and typically from 2 to 5. Advantageously, the plurality of microparticles is from 2 to 10 and typically from 2 to 4.

Other molecules may be coupled to the microparticle as long as they do not substantially prevent the carrier from being capable of enabling the complex to be transported to the circulation or lymphatic drainage system via the mucosal epithelium of the host. Such molecules include targetting molecules which target and attach the complex of the first embodiment to or in the vicinity of a desirable target in the host (eg an organ in the host). A carrier molecule which also functions as a targetting molecule may also be used. Examples of targetting molecules include antibodies (including monoclonal and polyclonal antibodies), lectins, enzymes, or other binding proteins or substances (or binding fragments thereof).

According to a third embodiment of this invention there is provided a composition for oral delivery of a substance or substances to the circulation or lymphatic drainage system of a host, comprising a mixture of a plurality of different complexes according to the first embodiment.

The complexes may be different in that the carrier, the microparticle and/or the substance of each complex may be different to the carrier, the microparticle and/or the substance of at least one of the other complexes.

The composition of the third embodiment can also include an acceptable carrier, diluent, excipient and/or adjuvant.

According to a fourth embodiment of this invention there is provided a composition for oral delivery of a substance to the circulation or lymphatic drainage system of a host, comprising the complex of the first embodiment together with a physiologically acceptable carrier, diluent, excipient and/or adjuvant.

According to a fifth embodiment of this invention there is provided a process for preparing a composition for oral delivery of a substance to the circulation or lymphatic drainage system of a host, comprising:

mixing a complex of the first embodiment with at least one different complex of the first embodiment.

The process of the fifth embodiment can further include mixing a physiologically acceptable carrier, diluent, excipient and/or adjuvant with the complex and the least one different complex.

A preferable composition of the fifth embodiment is a medicament comprising a carrier coupled to a microsphere or microcapsule comprising a hormone, drug, immunogen or DNA or RNA (such as ribozyme) component, molecule or analogues thereof in pharmaceutically active form.

According to a sixth embodiment of this invention there is provided a process for preparing a composition for oral delivery of a substance to the circulation or lymphatic drainage system of a host, comprising:

mixing the complex of the first embodiment with a physiologically acceptable carrier, diluent, excipient and/or adjuvant.

The nature of the carrier, diluent, excipient and/or adjuvant utilised in the composition of the third embodiment is dependent on the type of host. For instance, when the host is a human the carrier, diluent, excipient and/or adjuvant is pharmaceutically acceptable. When the host is non human such as an a non human mammal (eg a dog, cat, sheep, goat cow, bull, camel or horse) or other animal, the carrier, diluent, excipient and/or adjuvant is veterinarily acceptable.

10 Examples of pharmaceutically acceptable carriers, diluents and excipients for oral delivery include : sodium bicarbonate solutions and similar diluents which neutralise stomach acid or have similar buffering capacity, glycols, oils or emulsions; and include medicaments in the form of gels, pastes and viscous colloidal dispersions. The medicament may be presented in
15 capsule, tablet, slow release or elixir form or as a gel or paste. Furthermore the medicament may be presented as a food.

According to a seventh embodiment of this invention there is provided a method of orally delivering a substance to the circulation or lymphatic drainage system of a host requiring such substance, comprising:

20 orally administering to the host an effective amount of a complex of the first embodiment or a composition of the third or fourth embodiments.

A preferred method of the seventh embodiment is for treating a vertebrate host by administration of a hormone, drug, immunogen or DNA or RNA (such as ribozyme) component, molecule, analogue or derivative thereof
25 requiring such administration which method comprises the oral administration to the host of an effective amount of a carrier coupled to a microsphere or microcapsule comprising a hormone, drug, immunogen or DNA or RNA (such as ribozyme) component, molecule, analogue or derivative thereof appropriate to the therapy of the host.

30 According to an eighth embodiment of this invention there is provided a kit for preparing a complex for oral delivery of a substance to the circulation or lymphatic drainage system of a host, comprising:

at least one type of carrier;

at least one type of microparticle;

35 means to couple the microparticle to the carrier to form the complex;

the carrier being capable of enabling the complex to be transported to the circulation or lymphatic drainage system via the mucosal epithelium of the host;

the microparticle entrapping or encapsulating the substance whereby the substance is substantially unaffected by intestinal digestive substances of the host; and

the microparticle being adapted to release the entrapped or encapsulated substance into the circulation or lymphatic drainage system of the host.

The kit may include a plurality of the same or different carriers and/or a plurality of the same or different microparticles. The microparticles may contain the same substance or different substances. The kit may include at least one type of auxiliary molecule such as a targetting molecule and means to couple the auxiliary molecule(s) to the microparticle(s).

Hormones, drugs, immunogens or DNA or RNA (such as ribozyme) component, molecule or analogues thereof suitable to be incorporated within a microparticle, such as a microsphere or microcapsule include all hormones, drugs, immunogens or DNA or RNA (such as ribozyme) component, molecule or analogues thereof for which oral administration is desirable but for which oral administration in an unprotected form results in substantial loss of efficacy.

Thus typical substances for delivery according to the invention include active substances such as hormones and bioactive peptides (and analogues and derivatives thereof) such as LHRH, Vasopressin, oxytocin, Insulin, testosterone, interferon, somatotrophin, somatostatin, Erythropoietin, Colony Stimulating factors (G-CSF, GM-CSF, CSF), PMSG, HcG, Inhibin, PAI-II; therapeutic agents such as neomycin, salbutamol, pyrimethamine, penicillin G, methicillin, cabenicillin, pethidine, xylazine, ketamin HCl, mephensin, GABA, iron dextran, nucleotide analogues or ribozyme.

Further examples of active substances include polypeptides such as insulin, somatostatin, somatostatin derivatives (U.S. Pat. Nos. 4,087,390, 4,093,574, 4,100,117 and 4,253,998), growth hormones, prolactin, adrenocorticotrophic hormone (ACTH), melanocyte stimulating hormone (MSH), thyroid hormone releasing hormone (TRH), its salts, and derivatives thereof (U.S. Pat. Nos. 3,957,247 and 4,100,152), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), vasopressin, vasopressin derivatives [desmopressin [Folia Endocrinologica Japonica 54, No. 5, p. 676-691 (1978)]], oxytocin, calcitonin, parathyroid hormone, glucagon, gastrin, secretin, pancreozymin, cholecystokinin, angiotensin, human placental lactogen, human chorionic gonadotropin (HCG), enkephalin, enkephalin derivatives [U.S. Pat. No. 4,277,394, European patent application Publication No. 31567], endorphin, kyotorphin, interferons (a, b,

g), interleukins (I, II, and III), tuftsin, thymopoietin, thymosin, thymostimulin, thymic humoral factor (THF), serum thymic factor (FTS), and its derivatives (U.S. Pat. No. 4,229,438) and other thymic factors [Medicine in Progress 125, No. 10, p. 835-843 (1983)], tumor necrosis factor (TNF), colony stimulating factor (CSF), motilin, dinorphin, bombesin, neurotensin, cerulein, bradykinin, urokinase, asparaginase kallikrein, substance P analogue and antagonist, nerve growth factor, blood coagulation factors VIII and IX, lysozyme chloride, polymixin B, colistin, gramicidin, bacitracin, protein synthesis stimulating peptides (British patent No. 8232082), gastric inhibitory polypeptide (GIP), vasoactive intestinal polypeptide (VIP), platelet-derived growth factor (PDGF), growth hormone releasing factor (GRF, somatocrinin), bone morphogenetic protein (BMP), epidermal growth factor (EGF), etc.

Examples of antitumor agents include bleomycin hydrochloride, methotrexate, actinomycin D, mitomycin C, vinblastine sulfate, vincristine sulfate, daunorubicin hydrochloride, adriamycin, neocarzinostatin, cytosine arabinoside, fluorouracil, tetrahydrofuryl-5- fluorouracil, krestin, picibanil, lentinan, levamisole, bestatin, azimexon, glycyrrhizin, poly I:C, poly A:U and poly ICLC.

Examples of antibiotics, include gentamicin, dibekacin, kanandomycin, lividomycin, tobramycin, amikacin, fradiomycin, sisomicin, tetracycline hydrochloride, oxytetracycline hydrochloride, rolitetracycline, doxycycline hydrochloride, ampicillin, piperacillin, ticarcillin, cephalothin, cephaloridine, cefotiam, cefsulodin, cefmenoxime, cefmetazole, cefazolin, cefotaxime, cefoperazone, ceftizoxime, moxolactam, latamoxef, thienamycin, sulfazecin, and azthreonam.

The aforementioned antipyretic, analgesic and antiinflammatory drugs include, for instance, sodium salicylate, sulpyrine, sodium flufenamate, sodium diclofenac, sodium indomethacin, morphine hydrochloride, pethidine hydrochloride, levorphanol tartrate and oxymorphone. Examples of the antitussives and expectorants may be mentioned ephedrine hydrochloride, methylephedrine hydrochloride, noscapine hydrochloride, codeine phosphate, dihydrocodeine phosphate, alioclamide hydrochloride, chlophedianol hydrochloride, picoperidamine hydrochloride, cloperastine, protokylol hydrochloride, isoproterenol hydrochloride, salbutamol sulfate and terbutaline sulfate. Examples of sedatives include chlorpromazine hydrochloride, prochlorperazine, trifluoperazine, atropine sulfate and scopolamine methylbromide. The muscle relaxants include, among others, pridinol methanesulfonate, tubocurarine chloride and pancuronium bromide. The antiepileptics include, for instance, sodium phenytoin, ethosuximide, sodium

acetazolamide and chlordiazepoxide hydrochloride. Examples of antiulcer drugs include metoclopramide and L-histidine monohydrochloride. Examples of antidepressants include imipramine, clomipramine, noxiptiline and phenelzine sulfate. The antiallergic drugs include, among others, diphenhydramine hydrochloride, chlorpheniramine maleate, tripelenamine hydrochloride, methdilazine hydrochloride, clemizole hydrochloride, diphenylpyraline hydrochloride and methoxyphenamine hydrochloride. The cardiotonics include, among others, trans- p - oxocamphor, theophyllol, aminophylline and etilefrine hydrochloride. The antiarrhythmic agents include, for instance, propranolol hydrochloride, alprenolol hydrochloride, bufetolol hydrochloride and oxyprenolol hydrochloride. The vasodilators include, among others, oxyfedrine hydrochloride, diltiazem hydrochloride, tolazoline hydrochloride, hexobendine and bamethan sulfate. The antihypertensive diuretics include, among others, hexamethonium bromide, pentolinium, mecamlamine hydrochloride, ecarazine hydrochloride and clonidine hydrochloride. Examples of antidiabetics include sodium glymidine, glypizide, phenformin hydrochloride, buformin hydrochloride and metformin. The anticoagulants include, among others, sodium heparin and sodium citrate. The haemostatic agents include, among others, thromboplastin, thrombin, menadione sodium bisulfite, acetomenaphthone, 6-amino-caproic acid, tranexamic acid, carbazochrome sodium sulfonate and adrenochrome monoaminoguanidine methanesulfonate. Among antituberculotics are isoniazid, ethambutol and sodium p-aminosalicylate. The hormone drugs are exemplified by prednisolone succinate, prednisolone sodium phosphate, dexamethasone sodium sulfate, betamethasone sodium phosphate, hexestrol phosphate, hexestrol acetate and methimazole. The antinarcotic agents include, among others, levallorphan tartrate, nalorphine hydrochloride and naloxone hydrochloride.

Suitable carrier molecules include Vitamin B₁₂, a Vitamin B₁₂ analogue or derivative (as described in PCT/AU86/00299), or a lectin, or "lectin-like" molecule (such as that described in WO86/06635).

Suitable carrier molecules also include bacterial adhesins, viral adhesins, toxin binding subunits and lectins, as well as Vitamin B₁₂ and analogues thereof.

Analogues of Vitamin B₁₂ for use as carriers for microparticles include cyanocobalamin, aquocobalamin, adenosylcobalamin, methylcobalamin, hydroxycobalamin, cyanocobalamin carbanalide, 5-O-methylbenylcobalamin, and the desdimethyl, monoethylamide and methylamide analogues of all of the preceding analogues, as well as coenzyme B₁₂, 5'-deoxyadenosylcobalamin, chlorocobalamin, sulfitocobamin, nitrocobalamin, thiocyanatocobalamin, 5,6-

dichlorobenzimidazole, 5-hydroxybenzimidazole, trimethylbenzimidazole, adenosylcyanocobalamin, cobalamin lactone, cobalamin lactam, and analogues in which the cobalt is replaced by zinc or nickel or the corrin ring is replaced by a substituent which does not affect the binding capacity of the analogue to 5 IF.

Derivatives of Vitamin B₁₂ for use as carriers for microparticles include the anilide, ethylamide, monocarboxylic and dicarboxylic acid derivatives of Vitamin B₁₂ and its analogues as well as tricarboxylic acid or propionamide derivatives of Vitamin B₁₂ or its analogues. They would also include molecules 10 in which alterations or substitutions had been performed to the Corrin ring [viz:-cyano (13-epi) cobalamin Co a-(a 5,6-dimethylbenzimidazolyl)-Co, b-cyano-(13-epi) cobamic a,b,c,d,g, pentaamide, adenosyl-10-chlorocobalamin, dicyanobyrrinic heptamethyl ester, cyanoaquacobyrinic acid pentaamide], or where cobalt had been replaced by another metal ion (viz:- nickel, zinc, etc) or 15 various anion or alkyl substituents to the corrin ring such that the binding capacity of the molecule to intrinsic factor is unaffected. The mucosal epithelial cells will take up the intrinsic factor-vitamin B₁₂ complex including the microparticle, such as a microsphere or microcapsule attached to the vitamin B₁₂ (or suitable analogue) and transepithelially transport the 20 microsphere or microcapsule and deliver them into the circulation where the enclosed substance such as a hormone, drug, immunogen, or DNA or RNA (such as ribozyme) component, molecule or analogues thereof can act.

Derivatives and analogues of vitamin B₁₂ are discussed in Schneider, Z. and Stroinski, A.; *Comprehensive B₁₂*; Walter De Gruyter; Berlin, NY: 1987, 25 the disclosure of which is incorporated herein by reference.

Similarly, if a microparticle, such as a microsphere or microcapsule is administered orally and complexed to a carrier protein possessing binding activity to the mucosal epithelium, the cells of the mucosal epithelium take up those molecules including the microparticles, such as microspheres or 30 microcapsules attached to the carrier proteins and present the microsphere or microcapsule to the circulation where the substance such as a drug, hormone, immunogen or DNA or RNA (such as ribozyme) component, molecule or analogues thereof enclosed therein can act.

Polymers suitable for the formation of microspheres by solvent 35 evaporation (in liquid drying) include, amongst others, Poly-lactic acid, Poly-(Lactide/co-glycolide), Poly-hydroxybutyrate, Poly-hydroxyvalerate, Poly-(hydroxybutyrate/valerate), Ethyl cellulose, Dextran, Polysaccharides, Polyalkylcyanoacrylate, Poly-methyl-methacrylate, poly(ϵ -caprolactone) and various combinations and co-polymers of the above.

Polymers suitable for the formation of microspheres by interfacial precipitation/polymerization include, amongst others, EUDRAGITTM; Poly(N^a,N^b-L-lysinediylterephthaloyl); polymers formed by the reaction of Lysine hydrochloride and p-phthaloyl dichloride; by the reaction of acryloylated maltodextrin or acryloylated hydroxyethyl starch with ammonium peroxodisulfate and N,N,N',N'-tetramethylethylenediamine. Microspheres can also be formed by the polymerization of various diamines such as ethylene diamine, phenylenediamine, toluene diamine, hexamethylene diamine, or diols such as ethylene diol, bisphenol, resorcinol, catechol, pentanediol, hexanediol, dodecanediol, 1,4 butanediol, with diacid chlorides such as sebacoyl chloride and adipoyl chloride, or diisocyanates such as hexamethylene diisocyanate using the methods fully described in EP-A-85870002.4, the disclosure of which is incorporated herein by reference.

Polymers suitable for the formation of microspheres by polymer phase separation include co-poly(vinyl chloride:vinyl alcohol:vinyl acetate), cellulosic polymers, polyvinyl acetate, polyvinyl alcohol, polyvinylchloride, natural and synthetic rubbers, polyacrylates, polystyrene and the like. Methods and materials to synthesize such microspheres are fully described in US Pat. No. 4,166,800, the disclosure of which is incorporated herein by reference.

Polymers suitable for the formation of microspheres by complex coacervation include, amongst others, mixtures of polyanions, such as gum arabic, alginate, carboxymethyl cellulose, carboxymethyl starch, polystyrene sulfonic acid, polyvinyl sulfonic acid, poly-glucuronic acid, Poly-pyruvic acid, carrageenan, heparin sulphate, polyphosphate with polycations, such as polylysine, gelatin.

Polymers suitable for the formation of microspheres by Polymer/Polymer incompatibility include, amongst others, ethyl cellulose, Ethylene vinyl acetate polymer, Poly(lactide), or Poly(vinylidene chloride) mixed with polymers such as Polyethylene, Silicone, Polyisobutylene or Polybutadiene.

Other materials suitable for formation of microspheres include, Starch, Cross-linked Albumen, Polyacrylamide, Cross-linked gelatin and others obvious to those skilled in the art of microsphere preparation. Materials suitable for the formation of microspheres, and methods for the preparation of microspheres, are described in US Pat. Nos. 3,936,573 and 3,962,414, the disclosures of which are incorporated herein by reference.

According to the present invention there is also provided a process for the production of a complex of the invention, which process comprises one or more of the following steps :

(a) reacting microparticles with a carrier molecule to form the complex;

- (b) chemically modifying a carrier molecule to provide at least one functional group capable of forming a chemical linkage and reacting a microparticle and the modified carrier molecule to form the complex;
- (c) reacting microparticles with at least one cross-linking agent and reacting
5 the reacted microparticles with a carrier molecule to form the complex;
- (d) reacting a carrier molecule with at least one cross-linking agent and reacting microparticles with the reacted carrier molecule to form the complex;
- (e) reacting microparticles and a carrier with at least one cross-linking agent to form the complex;
- 10 (f) reacting microparticles with at least one cross-linking agent, reacting a carrier molecule with at least one cross-linking agent and reacting the reacted microparticles and the reacted carrier molecule to form the complex; or
- (g) reacting a carrier molecule with a hydrophobic moiety and reacting microparticles with the reacted carrier molecule to form a complex non-
15 covalently bonded by hydrophobic interaction.

As an example of reaction (g) above, in order to link Vitamin B₁₂ to the surface of microparticles which have no readily available chemical groups suitable for chemical conjugation, it is possible to prepare a complex of Vitamin B₁₂ to an hydrophobic moiety which can insert, non-covalently, into
20 the surface of the microparticles. Such a molecule is easily added at the time of formation of the microparticles. The strength of the hydrophobic association is such that there is only a very slow dissociation of the Vitamin B₁₂ from the microparticles under physiological conditions. Similarly, other carrier molecules may be reacted with hydrophobic moieties, for formation of an
25 hydrophobically-associated complex with a microparticle.

Suitable hydrophobic moieties which can be used for reacting with a carrier molecule are aliphatic or aromatic chains or amphipathics containing a water soluble head and a lipid soluble tail suitable for hydrophobic association within an hydrophobic environment. Examples include oleic acid, octanoic
30 acid, linoleic acid, stearic acid, palmitic acid or glycerophosphoric acids, which may be directly conjugated to an amino group of a carrier molecule using a suitable carbodiimide (for example dicyclohexylcarbodiimide (DCC), or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC)). Similarly, any amphiphathic molecule possessing an amino-group, for example amino-hexane, amino-
35 decane, amino-dodecane, amino-tetradecane, amino-hexadecane or phosphatidyl-ethanolamine, may be conjugated directly to carboxyl groups using carbodlimides.

Alternatively, the carrier molecule may be linked covalently, directly or indirectly to the microparticle. Where a cross-linking agent is used, the cross-

linking agent may contain a disulfide bond or be cleavable by acid, base or periodate. Examples of cross-linking agents include : N-(4-azidophenylthio)-phthalimide; 4,4'-dithiobisphenylazide; dithio-bis-(succinimidyl-propionate); dimethyl-3,3'-dithio-bis-propionimide.2HCl; 3,3'-dithio-bis-(sulfosuccinimidyl-propionate); ethyl-(4-azidophenyl)-1,3'-dithiopropionate; sulfo-succinimidyl-2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiobutyrimide.HCl; N-succinimidyl-(4-azido-phenyl)-1,3'-dithiopropionate; sulfo-succinimidyl-2-(m-azido-o-nitro-benzamido)-ethyl-1,3'-dithiopropionate; sulfo-succinimidyl-2-(p-azido-salicylamido)-ethyl-1,3'-dithiopropionate; N-succinimidyl-3-(2-pyridylthio)propionate; sulfosuccinimidyl-(4-azidophenyldithio)-propionate; 2-iminothiolane; disuccinimidyl tartrate; bis-[2-(succinimidylloxycarbonyloxy)-ethyl]-sulfone and carbodiimides. A description of suitable carbodiimides is provided in Khorana, H.G. (1953) Chem. Rev. 53: 145-166, the disclosure of which is incorporated herein by reference.

Examples of suitable methods of reacting vitamin B₁₂ (VB₁₂) derivatives with functionalised microparticles include:

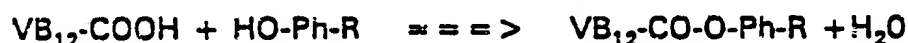
(i). Reaction of carboxy-VB₁₂ with amine

EDAC



(ii). Reaction of carboxy-VB₁₂ with phenol

EDAC



25

(iii) Reaction of amino-VB₁₂ with carboxylates

EDAC



Methods of coupling vitamin B₁₂ derivatives to various functional groups are also described in US Pat. No. 4,465,775, United Kingdom Patent No. 1,345,327 and US Pat. No. 3,981,863, the disclosures of which are incorporated herein by reference.

Suitable cross-linking of the carrier and the microspheres may be achieved by acid hydrolysis of the amide side groups of the propionamide side chains adjacent to rings A, B, C or D of Vitamin B₁₂ and coupling to suitable side groups of the microspheres.

The carrier molecule or cross-linking agent may react with a functional group or a modified functional group present on, or introduced onto the surface of the microparticle. Suitable functional groups for reaction with the

carrier molecule or cross-linking agent include carboxyl, hydroxyl, amino, thio, amido, hydrazo, azido, phenolic, ester, aldehyde, ketone, sulfate, halo, phosphate, isocyanato and isothiocyanato groups. Suitable reagents for modification or introduction of functional groups include hydrazine, periodate, permanganate or other oxidising agents, borohydrides, metallic hydrides or other reducing agents.

Alternatively, a spacer molecule may be used to link the carrier molecule to the microparticle. Examples of such spacer molecules include bifunctional molecules such as diamines, dicarboxylic acids, diols, aminocarboxylic acids, dithiols, diesters, diphenols, and other like molecules.

Advantageously, using a complex of the present invention, a substance such as a hormone, drug or immunogen can be presented via the mucosal epithelium of a host, in a pharmaceutically active form to the circulation or lymphatic drainage system of a host. Initially, microparticles such as microspheres or microcapsules, containing a substance such as a pharmaceutically active agent, are prepared and linked, generally covalently, to a suitable carrier (generally a mucosal binding protein or Vitamin B₁₂ or an analogue or derivative thereof) such that the carrier maintains its ability to interact with the intestinal mucosa or intrinsic factor (respectively). Then the microparticles are administered orally to a host and as a result of this administration the carrier-microparticles and the substance contained therein pass into the circulation or lymphatic drainage system of the host. In this fashion the substance is protected from the degradative contents of the intestinal milieu, and the uptake capacity of the carrier is amplified.

Thus, a complex according to the first embodiment of the present invention overcomes the disadvantages inherent in the mucosal binding protein and Vitamin B₁₂ uptake system, viz:- the need for substances, such as pharmaceutical agents, to be resistant to gastro-intestinal enzymes and pH conditions, as well as the limited uptake capacity of the uptake systems.

The present invention relies on the ability to entrap substances, which are generally small molecules, such as hormones, proteins, peptides, drugs, etc. within a matrix or capsule, generally fabricated from a suitable polymer, in such a way as to form very small microparticles such as microcapsules or microspheres. Once trapped within these microparticles it is possible using suitable chemistry to link, generally covalently link, these microparticles to a suitable carrier.

A system for oral delivery of an active substance coupled directly to Vitamin B₁₂ is limited in the amount of active substance that can be delivered by the uptake capacity of the IF-dependent uptake mechanism. In humans,

this mechanism can only deliver 1-2 μg doses of vitamin B₁₂ per feeding (see *Cobalamin. Biochemistry and Pathophysiology*. Ed Babior, B.M., Wiley-Interscience, NY, 1975.) Similarly, when microencapsulated active agents are administered orally, typically only from 0.1% to 1% of the active agent administered is delivered into the bloodstream (Gruber, R. Longer, M.A. and Robinson, K.J.R. 1987: *Some Biological Issues in Oral Controlled Drug Delivery*, Adv. Drug Delivery Rev. 1: 1-18).

Using carrier-microparticle complexes of the present invention, however, there is the potential to amplify the uptake of a substance administered orally, some 10 to one million times (depending upon the size of microparticle and the loading) as well as to protect the entrapped substance, typically a pharmaceutical agent, from intestinal digestive substances of the host, typically, gastrointestinal enzymes. By choosing a suitable substance for the microparticle such as a bio-degradable polymer the entrapped substance is released once the carrier mediated uptake system has delivered the carrier-microsphere complex to the circulation.

Amplification of Vitamin B₁₂ uptake capacity by the incorporation of pharmaceutical active agents into microspheres is illustrated in the following Table 1.

20

Table 1. Amplification of the Vitamin B₁₂ uptake capacity by the incorporation of pharmaceutically active agents into microspheres. Total delivery to man.

Microsphere diameter (nm)	Volume (cc)	Weight of microspheres ¹	Weight of pharmaceutical ²	Quantity delivered ³
.	.	.	1nm	0.001-0.01 nm
.	.	.	1nm + VB ₁₂	0.1 - 1 nm
20	4×10^{-18}	2.4 mg	240 μg	0.24 - 2.4 μg
20	4×10^{-18}	2.4 mg	240 μg + VB ₁₂	0.24 - 240 μg
200	4×10^{-15}	2.4 gm	240 mg	0.24 - 2.4 mg
200	4×10^{-15}	2.4 gm	240 mg + VB ₁₂	0.24 - 240 mg
2000	4×10^{-12}	2.4 kg	240 gm	0.24 - 2.4 gm
2000	4×10^{-12}	2.4 kg	240 gm + VB ₁₂	0.24 - 240 gm

1 Data is calculated from the uptake capacity for Vitamin B₁₂ of 1 nanomole per feed in man, which represents 6×10^{14} molecules of Vitamin B₁₂.

2 Each microsphere would be loaded to a 10 % drug loading.

3 With normal unassisted uptake approximately 0.1 - 1% of the dose of an orally administered pharmaceutical will cross the intestinal wall and enter the circulation. The Vitamin B₁₂ uptake mechanism has the capacity to amplify this uptake by at least one hundred fold.

A particular advantage of the carrier-microparticle complexes of the present invention compared with the carrier-active agent complexes of the prior art is that, there is no chemical modification of the active substance in the complexes of the present invention.

BEST MODE AND OTHER MODES FOR CARRYING OUT THE INVENTION

Microspheres containing a substance such as a hormone, drug, immunogen, or DNA or RNA (such as ribozyme) component, molecule or analogues thereof, are prepared typically by one or more of a number of techniques commonly known to those knowledgeable in the art, including :- Solvent evaporation, Complex coacervation, Polymer/polymer incompatibility, Gelation, Interfacial polymerization and Thermal denaturation.

For oral delivery microspheres are complexed with a carrier molecule by direct reaction or by use of cross-linking agents to provide a complex in which the carrier molecule is still able to undergo the binding reactions required for the uptake and transport of the complex and the pharmacological activity of the entrapped active substance is maintained. The carrier molecule is a mucosal binding protein or Vitamin B₁₂, or an analogue or derivative of Vitamin B₁₂ possessing binding activity to Castle's intrinsic factor.

A medicament containing an effective amount of the complex is formulated by mixing the complex with a pharmaceutically acceptable carrier, diluent, excipient and/or adjuvant. The medicament is prepared so as to be suitable for administration to a patient requiring treatment such as one or more of the conditions outlined in the body of the specification. The medicament is prepared using standard pharmaceutical techniques.

It is recognised that a number of factors will affect the determination of an appropriate dosage for a particular patient. Such factors include the age, weight, sex, general health and concurrent disease states of the patient. The determination of the appropriate dose level for the particular patient is performed by standard pharmaceutical techniques.

The medicament is orally administered to the patient in an amount such that an appropriate effective dosage of the substance in the complex

contained in the medicament is delivered to the circulation or lymphatic drainage system of the patient.

The invention is further described with reference to the following 5 examples which are in no way limiting on the scope of the invention. Throughout the following examples, reference to "VB₁₂" is to be taken as reference to Vitamin B₁₂.

EXAMPLE 1

Preparation of microspheres by Coacervation

10 Almost any protein can be used as the matrix for entrapping drug via the desolvation technique, however preferred proteins according to the invention include bovine serum albumen (BSA), Ovalbumen (OA), collagen,

Microspheres were prepared by coacervation of BSA following desolvation, according to the method of Oppenheim (Oppenheim, 1984, 15 Oppenheim et al 1984, 1982), Briefly a 40% ammonium sulphate solution was added dropwise to a solution of 1% BSA containing 0.5% Tween 20 and the turbidity monitored by Klett readings, until the turbidity rose rapidly. At this point (determined by experimentation) the solution was placed in an ultraturrax and 600 ul of glutaraldehyde added to cross-link the nanoparticles. 20 Cross-linking was stopped by the addition of a solution of 12% sodium metabisulfite.

Particles were then washed extensively with distilled water prior to coupling to the amino-derivative of Vitamin B₁₂.

EXAMPLE 2

Incorporation of Neomycin Sulphate

For incorporation of the antibiotic, neomycin sulphate, neomycin sulphate was dissolved at 10 g/100 ml of the BSA/Tween solution. Desolvation and cross-linking was carried out as described in Example 1.

30

EXAMPLE 3

Preparation of Insulin Microspheres

Insulin microspheres were prepared in a similar fashion to the BSA microspheres except the initial desolvation was achieved by the dropwise 35 addition of 0.1 N HCl, while resolution was achieved by the addition of 0.1 N NaOH.

EXAMPLE 4**Coupling of microspheres to amino-ethyl-Vitamin B₁₂**

The monocarboxylic acid derivative of Vitamin B₁₂ was prepared as previously described by Allen and Majerus (1972). The diamino-ethane derivative of COOH-Vitamin B₁₂ was prepared by reacting N,N-dicyclohexyl carbodiimide with a solution of diaminoethane (pH 6.5). The amidated derivative was purified by HPLC.

Proteinaceous microspheres were coupled to amino-ethyl Vitamin B₁₂ by reaction with N,N-dicyclohexyl carbodiimide.

EXAMPLE 5**Oral feeding**

The VB₁₂-microsphere complex can be administered orally by feeding in a solution of 0.1 M carbonate buffer pH 9.5.

Uptake of the VB₁₂-microspheres occurs via the intrinsic factor mediated VB₁₂ uptake mechanism.

EXAMPLE 6**Preparation of VB₁₂-Lipid complexes for hydrophobic insertion into microspheres****a) Preparation of VB₁₂-phosphatidyl ethanolamine (VB₁₂-PEA)**

Phosphatidylethanolamine (PEA, 100mg) was dissolved in 2 ml chloroform/methanol (50:50, v/v). Monocarboxyl VB₁₂ ("e" isomer) (100 mg) was added to the mixture. The monocarboxylic acid isomer was then cross-linked to the PEA by the addition of 200 mg of the carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC or EDAC). The reaction was allowed to proceed for 90 minutes prior to the addition of the VB₁₂-PEA to microspheres.

b) Preparation of other complexes between VB₁₂ and an hydrophobic moiety

Covalent complexes can be made between analogues of VB₁₂ and almost any aliphatic or aromatic chains or amphipathic molecule containing a water soluble head group suitable for conjugation and a lipid soluble tail suitable for hydrophobic association within an hydrophobic environment. Thus, any lipid (saturated, unsaturated or polyunsaturated) which has a carboxylic acid head group, such as Oleic acid, octanoic acid, linoleic acid or glycerophosphoric acids may be directly conjugated to an amino-VB₁₂ derivative using a suitable carbodiimide (EDAC or dicyclohexylcarbodiimide, for example). Similarly any amphipathic molecule possessing an amino-group (amino-hexane, amino-

decane, amino-dodecane, phosphatidyl-ethanolamine) may be conjugated directly to carboxy-VB₁₂ using carbodiimides.

EXAMPLE 7

5 Preparation of VB₁₂-Microspheres by solvent evaporation

a) Preparation of VB₁₂-PEA-[Polymethylmethacrylate] microspheres

Polymethylmethacrylate (PMM, Polysciences)(MW 12,000; 500mg) was dissolved in 2 ml of dichloromethane (DCM). The PMM in DCM was then added dropwise to 20 ml of 0.25% Polyvinylalcohol (PVA) while homogenizing 10 at 13,500 rpm with a Janke & Kunkel Ultraturrax. After 1 minute, 200 μ l of VB₁₂-PEA was added and stirred gently overnight. The pink microspheres were then harvested by centrifugation, washed three times with water and lyophilized.

15 b) Preparation of VB₁₂-[PEA-Poly-lactic acid] microspheres

Poly-lactic acid (PLA, Polysciences)(MW 50,000; 500mg) was dissolved in 3 ml of DCM and then homogenized into 20 1% PVA at 13,500 rpm on an Ultraturrax T25 with an S25F probe for 5 minutes. VB₁₂-PEA (400 μ l) was added while the solution was stirred gently. Microspheres were harvested as 20 described above.

c) Preparation of VB₁₂-PEA-[Poly-Hydroxy-butyrate/valerate] microspheres

Poly-Hydroxy-butyrate/valerate (9% valerate) (ICI; 500 mg) was dissolved in 5 ml of DCM and homogenized into 20 ml 1% PVA at 13,500 rpm 25 on an Ultraturrax T25 with an S25F probe for 5 minutes. VB₁₂-PEA (400 μ l) was added and the spheres processed as described in 8b.

EXAMPLE 8

Covalent conjugation of VB₁₂ to microspheres with surface carboxyl groups

30 A general method for the conjugation of VB₁₂ to the surface of microspheres made from polymers with free carboxyl groups is outlined below. The specific example utilizes commercially available carboxyl-modified microspheres.

Polysciences FluoresbriteTM carboxylate Microspheres (2.5% Solids Latex) 35 were obtained from Polysciences in sizes of 0.045 μ m, 0.49 μ m, 2.2 μ m and 9.97 μ m. One ml of each of the preparations was washed extensively with distilled (DW) and resuspended in 200 μ l of distilled water. To each preparation was added 1.5 mg aminododecyl VB₁₂ then 5 mg of EDAC. Each

preparation was allowed to react overnight, after which unreacted material was removed by repeated washing with DW or by dialysis against DW.

EXAMPLE 9

5 Surface derivatization of microspheres

Many polymers used in the preparation of microspheres by solvent evaporation do not contain functional groups for direct conjugation to VB₁₂ or its functionalized analogues, however it is possible to modify the surface of the preformed microspheres to introduce functional groups suitable for
10 conjugation to VB₁₂.

a) Surface derivatization of Polylactic acid (PLA) microspheres

A preformed PLA microspheres (10 mg) were gently suspended in DW (350 μ l) by rotation on a rotary shaker for 2 hours. Hydrazine hydrate (10 μ l) was added and the suspension was shaken overnight at room temperature.
15 The spheres were spun down and repeatedly washed with water by re-suspension and centrifugation. The washing procedure was repeated until the supernatant failed to give a positive hydrazine test (purple colour upon reaction with a solution of trinitrobenzenesulfonate; 1 mg /ml). The spheres were washed a further two times and the wet pellet used directly for
20 conjugation to VB₁₂.

b) Conjugation of VB₁₂ to hydrazine modified PLA microspheres

A sample of the hydrazine modified PLA microspheres (3 μ l wet pellet) was suspended in DW (250 μ l). Aqueous solutions of the "e" monocarboxylic acid isomer of VB₁₂ ("e"CB₁₂) (10 mg/ml, 400 μ l) and EDAC (100 mg/ml, 100
25 μ l) were added and the reaction mixture shaken overnight at room temperature. The suspension was spun down and the supernatant removed. The pellet was washed repeatedly with DW (6 washes). The residual pellet, which was pale pink in colour, was vacuum dried prior to measurement in the IF assay.

30 Two control reactions were performed concurrently with the above conjugation. In the first a 3 mg sample of hydrazine-modified PLA microspheres was treated with the "e"CB₁₂ as described above but DW was used in place of the EDAC solution. In the second control a 2 mg sample of unmodified PLA microspheres was treated with both "e"CB₁₂ and EDAC as
35 described above. For both controls the pellet remaining after repeated washing was a clear white colour with no evidence of any associated VB₁₂.

EXAMPLE 10**Intrinsic Factor binding assay**

The ability of various VB₁₂-microsphere preparations to bind to porcine intrinsic factor was assessed in an intrinsic factor binding assay.

5 VB₁₂ and VB₁₂-microsphere preparations were diluted out in six-tenfold dilutions in IF buffer (1 mg/ml BSA [B₁₂ and IF deficient; Sigma #A-3902] in 0.1 M Phosphate buffer pH7.5). To 225 μ l of IF buffer was added 25 μ l of the above dilutions. Co⁵⁷VB₁₂ (0.25 ml, 0.25 ng in IF buffer) was then added to each sample. Porcine IF (0.25 ml; 1 IU/ml in IF buffer) was then added and the
10 material allowed to incubate at RT for 20 min. BSA-coated charcoal (0.25 ml; 0.5% BSA [B₁₂ and IF free] plus 2.5% charcoal) was added to each sample, vortexed and then centrifuged. The supernatant from each sample was then counted on a gamma counter set for counting Co⁵⁷. Results were determined as a percentage inhibition of the Co⁵⁷-VB₁₂ binding.

15

EXAMPLE 11**Estimation of IF binding activity of VB₁₂ microspheres**

Microspheres prepared with VB₁₂ surface coating were examined for IF binding as described above. The percentage binding is presented in the table
20 below.

Table 2. IF binding of various VB₁₂-microsphere preparations.

2a. IF binding by VB-Carboxylate microspheres (See Example 8)

25

<i>Microsphere preparation</i>	<i>MS weight</i>	<i>% inhibition of binding¹</i>
Carboxylate 9.97 μ m	0.625mg	27%
Carboxylate 1.87 μ m	62.5 μ g	62%
Carboxylate 0.49 μ m	6.25 μ g	40%
Carboxylate 0.045 μ m	0.625 μ g	90%

¹ Data is presented as the percentage inhibition of binding of Co⁵⁷B₁₂ to 2 U IF.

2b. IF binding by VB₁₂-PEA coated microspheres

Microsphere preparation	Microsphere weight ²
VB ₁₂ -PEA-PMM microspheres ³	140 µg
VB ₁₂ -PEA-PLA microspheres ³	100 µg
VB ₁₂ -PEA-PHB/PHV microspheres ³	75 µg
"e"VB ₁₂ -hydrazide-PLA microspheres ⁴	100 µg

² Data is expressed as the weight of microspheres which could showed
5 equivalent IF binding as 10 ng of VB₁₂.

³ Microspheres prepared as in Example 8.

⁴ Microspheres prepared as in Example 9.

EXAMPLE 1210 Covalent conjugation of Mucosal Immunogens to Fluorescent microspheres

Amino-ethyl derivatized Polysciences Fluoresbrite™ carboxylate
Microspheres (2.5% Solids Latex) in sizes of 0.045µm, 0.49µm, 2.2µm and
9.97µm were prepared by the addition of 500 µl of 0.1 M diaminoethane pH
6.5 to 2 ml of spheres suspended to 2.5%. Surface modification was then
15 obtained by the addition of 50 mg of dry EDAC to each preparation. Unreacted
material was removed by centrifugation and washing with DW. Finally
microspheres were resuspended in 3 ml of DW. The spheres were then
separated into 3 X 1 ml aliquots and treated as follows :-

a) Conjugation to LTB

20 Amino-ethyl microspheres were activated with glutaraldehyde by the addition
of 40 µl of a 25% solution of glutaraldehyde plus 100 µl of 0.1 M sodium
phosphate buffer pH 6.5. After reaction for 20 minutes at room temperature,
100 µl of 1 M HCL was added to the spheres which were then washed twice
by centrifugation and resuspension in 10 mM HCl. Finally the spheres were
25 resuspended in 1 ml of DW. LTB (2 mg in 1 ml 0.1 M carbonate buffer pH 9.5)
was then added and allowed to conjugate to the activated microspheres
overnight. Finally the Schiff's base formed during the conjugation was
stabilized by reduction with 200 µl of cold sodium borohydride for two hours

on ice. The microspheres were then washed 3 times in 0.1 M carbonate buffer, pH 9.5, and resuspended in 500 μ l of the same buffer. Microspheres were then stored at 4°C until used for oral feeding.

b) Conjugation to K99 pili

- 5 Glutaraldehyde activated amino-ethyl microspheres (prepared as described in Example 13a) were conjugated to K99 pili by the addition of 2 ml of K99 pili (1 mg/ml) plus 100 μ l of 0.1 M carbonate buffer and reaction overnight at room temperature.

The Schiff's base was reduced and the microspheres washed as described in 10 Example 12a.

c) Conjugation to 987P pili

Amino-ethyl microspheres (1 ml) were conjugated to 987P pili (2 mg in 200 μ l DW) by the addition of 20 mg of EDAC. After reaction overnight the spheres were washed with 0.1 M carbonate buffer, pH 9.5, as described previously.

15

Example 13

Oral administration of Fluoresbrite Microspheres conjugated to VB₁₂, 987P, K99 and LTB

- Fluoresbrite Microspheres conjugated to VB₁₂, 987P, K99 and LTB were orally 20 administered to conscious mice using a suitable feeding needle. At various times after oral administration the mice were killed by cervical dislocation and the small intestines removed surgically. The intestinal contents were then removed by washing the intestines with a solution containing 0.1 % Tween 20 in 0.1 M phosphate buffer pH 7.4. The small intestine was then cut into four 25 sections, filled with embedding media and frozen prior to sectioning in a cryostat. Sections were examined by light microscopy using a ZEISS fluorescent microscope.

- Close examination of sections obtained from mice fed microspheres conjugated to either VB₁₂, 987P, K99 or LTB revealed very similar patterns of 30 binding of spheres to the tips of intestinal epithelial cells. Microspheres of sizes 0.047 μ m, 0.45 μ m and 1.87 μ m could be seen clearly adhering to the tips of the epithelial cells within 2 hours of feeding, regardless of which molecule the microspheres were coated with. The pattern of binding varied somewhat depending upon the coating of the microspheres, thus VB₁₂ coated 35 microspheres were found to bind mainly to the cells of the ileum and lower jejunum, while microspheres coated with LTB were found to bind down the entire length of the small intestine. Microspheres coated with either 987P pili or K99 pili were found to bind most predominantly in the jejunum. In some

sections, microspheres of up to 0.45 μm appeared to have entered the body of the epithelial cell.

Example 14

5 Oral Administration of PLA Microspheres containing ^{125}BSA and coated with $\text{VB}_{12}\text{-PEA}$

Two preparations of PLA microspheres were synthesized as described previously. Prior to synthesis ^{125}BSA was added to the PLA in DCM. $\text{VB}_{12}\text{-PEA}$ was added to one of the preparations during the solvent evaporation step.
 10 Solvent was evaporated overnight, after which the microspheres were washed extensively with distilled water. Microspheres suspended in 0.1% BSA in saline were then fed to female Swiss mice. At various times after feeding, the mice were bled from the retro-orbital plexus and 125 counts determined in a Beckman gamma counter.

15

Table 3. Uptake of ^{125}BSA incorporated into PLA spheres or PLA spheres coated with $\text{VB}_{12}\text{-PEA}$

Microsphere preparation	Counts in the blood*		
	T60	T150	T240
PLA	0.76 ± 0.19	0.56 ± 0.02	0.51 ± 0.02
PLA + $\text{VB}_{12}\text{-PEA}$	1.61 ± 0.14	1.15 ± 0.01	1.29 ± 0.02
p-value	<0.01	<0.01	<0.01

* Counts are represented as the percentage of counts released from the stomach of mice fed the various microsphere preparations. The data are
 20 presented as the average of three mice \pm 1 standard deviation.

As can be seen from the data, there was a highly significant increase in the amount of BSA which was taken up into the blood in mice fed $\text{VB}_{12}\text{-PEA}$ microspheres in comparison to those fed the PLA spheres alone.

25

INDUSTRIAL APPLICABILITY

The present invention provides a simple and novel technique for the specific protection of active substances comprised within a complex during their transit down the intestine, prior to Intrinsic Factor or mucosal binding protein mediated uptake of the complexes. The invention also provides a
 30 method for the amplification of the VB_{12} uptake system. Thus the present invention provides a simple and novel technique for the specific protection of active substances from enzymatic degradation as well as for amplification of

the VB₁₂ uptake system thus enabling a wide range of active agents to be actively absorbed intact from the intestine.

REFERENCES

- 5 Oppenheim R.C. (1984) in "Polymeric Microparticles" (Guiot, P and Couvreur, P. Eds.) CRC Press, Boca Raton.
Oppenheim R.C., Gipps, E.M. Forbes, J.F. and Whitehead R.H. (1984) in "Microspheres and Drug Therapy" (Davis, S.S., Illum, L., McVie, J.G. and Tomlinson, E. Eds) Elsevier Science Publishers B.V.
- 10 Oppenheim, R.C., Stewart, N.F., Gordon, L. and Patel, H.M. (1982) Drug Devel. Indust. Pharm. 8: 531-546.
Allen, R.H. and Majerus, P.W. (1972) J.Biol. Chem. 247: 7702-7717.

Claims:

1. A complex for oral delivery of a substance to the circulation or lymphatic drainage system of a host, comprising:
 - a microparticle coupled to at least one carrier;
 - 5 the carrier being capable of enabling the complex to be transported to the circulation or lymphatic drainage system via the mucosal epithelium of the host;
 - the microparticle entrapping or encapsulating the substance whereby the substance is substantially unaffected by intestinal digestive substances of
 - 10 the host; and
 - the microparticle being adapted to release the entrapped or encapsulated substance into the circulation or lymphatic drainage system of the host.
2. A complex for oral delivery of a substance to the circulation or
15 lymphatic drainage system of a host, comprising:
 - a microparticle coupled to at least one carrier;
 - the carrier being capable of enabling the complex to be transported to the circulation or lymphatic drainage system via the mucosal epithelium of the host;
 - 20 the microparticle being capable of entrapping or encapsulating the substance whereby the substance is substantially unaffected by intestinal digestive substances of the host; and
 - the microparticle being adapted to release the entrapped or encapsulated substance into the circulation or lymphatic drainage system of
 - 25 the host.
3. A complex according to claim 1, wherein the microsphere or microcapsule entraps or encapsulates a hormone, drug, immunogen, or DNA or RNA (such as ribozyme) component, molecule or analogue thereof.
4. A complex according to claim 2, wherein the microsphere or
30 microcapsule is capable of entrapping or encapsulating a compound selected from the group consisting of a hormone, drug, immunogen, DNA component, RNA component, DNA molecule, RNA molecule and analogues thereof.
5. A complex according to claim 1, wherein the carrier molecule is selected from the group consisting of a mucosal binding protein, a bacterial adhesin, a
35 viral adhesin, a toxin binding subunit, a lectin, Vitamin B₁₂ and an analogue of Vitamin B₁₂ possessing binding activity to Castle's intrinsic factor.
6. A complex according to claim 2, wherein the carrier molecule is selected from the group consisting of a mucosal binding protein, a bacterial adhesin, a

viral adhesin, a toxin binding subunit, a lectin, Vitamin B₁₂ and an analogue of Vitamin B₁₂ possessing binding activity to Castle's intrinsic factor.

7. A complex according to claim 5 or 6 wherein the carrier is selected from the group consisting of Vitamin B₁₂, an analogue of Vitamin B₁₂,
5 possessing binding activity to Castle's intrinsic factor, a lectin, a pilum, a viral haemagglutinin and a toxin binding subunit.

8. A complex according to claim 1, wherein the microparticle further comprises a targetting molecule, wherein the targetting molecule is capable of targetting and attaching said complex to a target in a host.

10 9. A complex according to claim 2, wherein the microparticle further comprises a targetting molecule, wherein the targetting molecule is capable of targetting and attaching said complex to a target in a host.

10. A complex according to claim 8 or 9, wherein the targetting molecule is an antibody, lectin, enzyme, binding protein or binding substance, or a binding
15 fragment of an antibody, lectin, enzyme, binding protein or binding substance.

11. A complex according to claim 1 or 2, wherein the microparticle is coupled to a plurality of carriers.

12. A complex according to claim 1 or 2, wherein each microparticle has one carrier coupled thereto.

20 13. A complex according to claim 1 or 2, wherein the coupling is by means of covalent bonding or hydrophobic interaction.

14. A complex according to claim 13, wherein the covalent bonding is by a cross-linking agent.

15. A composition for oral delivery of a substance or substances to the
25 circulation or lymphatic drainage system of a host, comprising a mixture of a plurality of different complexes according to claim 1.

16. A composition according to claim 15, further comprising a physiologically acceptable carrier, diluent, excipient or adjuvant.

17. A composition for oral delivery of a substance to the circulation or
30 lymphatic drainage system of a host, comprising the complex of claim 1 together with a physiologically acceptable carrier, diluent, excipient or adjuvant.

18. A composition according to claim 16 or 17, wherein the carrier, diluent, excipient or adjuvant is orally and pharmaceutically acceptable.

35 19. A method of orally delivering a substance to the circulation or lymphatic drainage system of a host requiring such substance, comprising:

orally administering to the host an effective amount of a complex according to claim 1.

20. A method of orally delivering a substance to the circulation or lymphatic drainage system of a host requiring such substance, comprising:

orally administering to the host an effective amount of a complex according to claim 2.

5 21. A method according to claim 19 or 20, wherein the host is selected from the group consisting of a vertebrate host, a mammal and a human.

22. A method according to claim 19 or 20, wherein said complex comprises at least one carrier coupled to a microsphere or microcapsule, said microsphere or microcapsule comprising a compound selected from the group
10 consisting of a hormone, drug, immunogen, DNA component, RNA component, DNA molecule, RNA molecule and analogues thereof.

23. A process for the production of a complex according to claim 1 or 2, which process comprises one or more of the following steps:

(a) reacting microparticles with a carrier molecule to form the complex;

15 (b) chemically modifying a carrier molecule to provide at least one functional group capable of forming a chemical linkage and reacting a microparticle and the modified carrier molecule to form the complex;

(c) reacting microparticles with at least one cross-linking agent and reacting the reacted microparticles with a carrier molecule to form the complex;

20 (d) reacting a carrier molecule with at least one cross-linking agent and reacting microparticles with the reacted carrier molecule to form the complex;

(e) reacting microparticles and a carrier with at least one cross-linking agent to form the complex;

(f) reacting microparticles with at least one cross-linking agent, reacting a
25 carrier molecule with at least one cross-linking agent and reacting the reacted microparticles and the reacted carrier molecule to form the complex; or

(g) reacting a carrier molecule with a hydrophobic moiety and reacting microparticles with the reacted carrier molecule to form a complex non-covalently bonded by hydrophobic interaction.

30 24. A kit for preparing a complex for oral delivery of a substance to the circulation or lymphatic drainage system of a host, comprising:

at least one type of carrier;

at least one type of microparticle;

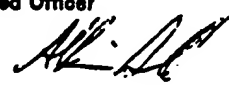
means to couple the microparticle to the carrier to form the complex;

35 the carrier being capable of enabling the complex to be transported to the circulation or lymphatic drainage system via the mucosal epithelium of the host;

the microparticle entrapping or encapsulating the substance whereby the substance is substantially unaffected by intestinal digestive substances of the host; and

the microparticle being adapted to release the entrapped or
5 encapsulated substance into the circulation or lymphatic drainage system of the host.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶																						
According to International Patent classification (IPC) or to both National Classification and IPC Int. Cl. ⁸ A61K 9/50, 9/52, 47/24, 47/42, 47/46, 47/48																						
II. FIELDS SEARCHED																						
Minimum Documentation Searched ⁷																						
Classification System	Classification Symbols																					
IPC	A61K 9/50, 9/52, 47/00, 47/24, 47/42, 47/46, 47/48																					
Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸																						
AU : IPC as above																						
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹																						
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate of the relevant passages ¹²	Relevant to Claim No ¹³																				
X	EP,A, 36277 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 23 September 1981 (23.09.81). See claims, abstract, page 2 line 24 - page 3 line 30, page 19.	1-4, 8-10, 12, 13, 17-18, 24																				
X	WO,A, 88/00474 (GEHO, W. Blair and LAU, John R) 28 January 1988 (28.01.88). See claims.	1-4, 8-9, 12-14, 17-24																				
X	WO,A, 88/07365 (RANNEY, David F) 6 October 1988 (06.10.88), see claims, page 3 line 30 - page 4 line 22	1-4, 8-10, 12-13, 17-22, 24																				
A	AU,B, 79929/87 (607439) (SOUTHERN RESEARCH INSTITUTE; THE UAB RESEARCH FOUNDATION), 28 April 1988 (28.04.88)																					
(continued)																						
<p>Special categories of cited documents : ¹⁰</p> <table border="0"> <tr> <td>"A"</td> <td>Document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier document but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"Z"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	Document defining the general state of the art which is not considered to be of particular relevance	"T"	Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"Z"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	Document defining the general state of the art which is not considered to be of particular relevance	"T"	Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"Z"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
IV. CERTIFICATION																						
Date of the Actual Completion of the International Search 3 July 1992 (03.07.92)		Date of Mailing of this International Search Report 16 July 1992 (16.07.92)																				
International Searching Authority AUSTRALIAN PATENT OFFICE		Signature of Authorized Officer ALBIN SMRDEL 																				

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A

**WO/A, 89/08449 (SOUTHERN RESEARCH INSTITUTE; THE
UAB RESEARCH FOUNDATION) 21 September 1989 (21.09.89).**

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..., because they are dependant claims and are not drafted in accordance with the second and third sentences of PCY Rule 6.4a

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 92/00141

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
EP	36277	AT	15140	CA	1165238
		US	4598051	US	4808466
		EP	91958	IT	8249401
		WO	8301571	AU	16275/88
		US	4925678	US	5108759
DE				DE	3171973
				CA	1198053
				IT	1149111
				EP	352295
				JP	58501819
WO	88/00474	AU	61413/86	DK	1256/88
		EP	274467	JP	1500747
FI				FI	881042
				NO	881058
WO	88/07365	AU	16275/88	EP	352295
		US	5108759	US	4925678
AU	79929/87	DK	5577/87	EP	266119
		JP	63190833	KR	9003559
		ZA	8707946	US	5075109
		AU	33433/89	CN	1043442
		EP	333523	IL	89602
		JP	3503892	NZ	228376
		ZA	8902103	IL	84167
				NZ	222278
WO	89/08449	US	5075109	AU	33433/89
		DK	2224/90	EP	333523
		IN	169330	JP	3503892
		ZA	8902103	AU	79929/87
		EP	266119	IL	84167
		KR	7003559	NZ	222278
		CN	1036326	CN	1043442
				IL	89602